New Variant of Low Density Lipoprotein Receptor Gene

FH-Tonami

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A new variant of the low density lipoprotein receptor (LDLR) gene was ascertained through Southern blotting analysis of LDLR genes of 35 unrelated Japanese patients with heterozygous familial hypercholesterolemia (FH). This mutant gene had a 6 kilo-base deletion which had eliminated only exon 15, an exon that encodes the O-linked sugar domain. The mutation was recognized in two patients with heterozygous FH. We refer to these patients as 'FH-Tonami', since they were both born in the Japanese district of Tonami. Although there is no evidence of a relation between families, the possibility of a common ancestor with FH does exist. Neonatal diagnosis of FH in two fetuses from one family was possible through analyses of their LDLR genes in cord blood samples at delivery. (Arteriosclerosis 8:187–192, March/April 1988)

A number of scientists have attempted to ascertain gene mutations in genetic diseases and to diagnose diseases with molecular biological analysis.1,2 Mutations in the DNA interest region can be detected by several methods; direct genomic sequencing, detection of gross structural alteration of the gene, linkage analysis using restriction fragment length polymorphisms (RFLPs), and others. Southern blotting analysis3 of genomic DNAs can not only visualize large insertions or deletions of the gene, but can also uncover one or more base-pair mutations destroying a common restriction site or producing a new site.

Mutations in the low density lipoprotein receptor (LDLR) gene produce familial hypercholesterolemia (FH),4 which is characterized by elevated serum LDL cholesterol levels, tendon xanthomas, and premature coronary artery disease.5,6,7

Since 1984 when the full length cDNA for human LDLR was cloned,8 it has been possible to analyze the abnormalities of LDLR genes with molecular biological techniques. Brown, Goldstein, and colleagues cloned genomic DNA of the LDLR gene9 and revealed several gene mutations of LDLR in FH patients.10-18 One FH patient19 had inherited two different mutations from his parents. From his mother he had inherited a mutant gene that produced no detectable receptor (null allele). Recent evidence has uncovered a 5 kilo-base (kb) deletion in this mutant allele produced by exon-Alu recombination.12 From his father this patient had inherited a mutant gene encoding the internalization-defective receptor. This mutant allele had a point mutation in the codon corresponding to amino acid 807 in the cytoplasmic tail of the LDLR which caused the substitution of a cysteine for tyrosine.13

Humphries and his colleagues20,21,22 and Langlois et al.23 have also reported FH cases with large deletions in the LDLR gene.

In Lebanon a nonsense mutation producing truncated LDLR was identified.16 The analyses revealed that four unrelated Arab patients with homozygous FH had two copies of this mutant gene. The mutation was believed to be a type common to this district and was referred to thereafter as the 'Lebanese allele'.

In this study, we investigated LDLR genes with cloned human LDLR cDNA to find their mutations in Japanese FH patients. Through the analyses of 35 unrelated heterozygous FH patients, an approximately 6 kb deletion in the LDLR gene was identified in two cases (KY and SO). This mutant gene proved to be a new type which had lost exon 15, an exon that encodes the O-linked sugar domain, and parts of two adjacent introns. Because these two patients had the same mutant gene, we believe that they may have descended from a common ancestor with FH ('FH-Tonami'). In SO's family, two newborn babies were diagnosed as having FH by the presence or absence of this 6 kb-deleted abnormal LDLR gene.

Methods

Patients who visited Kanazawa University Hospital and its affiliated hospitals were diagnosed as having FH according to the following criteria: primary hypercholesterolemia (above 230 mg/dl) with tendon xanthomas in the patient24 and primary hypercholesterolemia with or without tendon xanthomas in a first-degree relative.

High molecular genomic DNA samples were isolated from 10 ml peripheral blood by a modified Triton X-100
lysis method. After digestion with restriction enzymes (Takara Shuzo, Kyoto, Japan), DNA fragments were separated by electrophoresis through 0.8% to 1.0% agarose gel and were transferred to a nitrocellulose filter (Schleicher and Schuell, Dassel, W. Germany) by the Southern blotting method. Filters were prehybridized for several hours at 42°C in 50% formamide, 3 x SSC (20 x SSC is 0.3 M sodium citrate, 3 M NaCl), 5 x Denhardt (50 x Denhardt's solution is 1 g Ficoll, 1 g polyvinylpyrolidone, 1 g bovine serum albumin per 100 ml), 20 μg/ml denatured salmon sperm DNA (Sigma Chemical Company, St. Louis, Missouri). Then, with the labelled cDNA probe at a concentration of 10 ng/ml, filters were hybridized for 20 hours at 42°C in 50% formamide, 3 x SSC, 5 x Denhardt, 20 μg/ml denatured salmon sperm DNA, and 100 mg/ml dextran sulfate. A few posthybridization washes were carried out at 60°C in 0.4% sodium dodecyl sulfate, 0.4 x SSC for 15 minutes. After that, filters were autoradiographed for several days on Fuji x-ray film (Fuji Photo Film Company, Kanagawa, Japan) by using an intensifying screen at -70°C.

Full length cDNA (pLDLR-3) and partial cDNA (pLDLR-2HHI) for human LDLR were kind gifts from David W. Russell. The probe pLDLR-2HHI consists of a 1.9 kb fragment of the 3' end of the pLDLR-3 and has been cloned into pSP64. This pLDLR-2HHI fragment was isolated by 1% agarose gel electrophoresis after digestion with the restriction enzyme, BamHI. Because the pLDLR-3 contains repetitive Alu sequences, those sequences were removed by XbaI and Smal digestion. This new probe, designated as pLDLR-3XSI, had a 2.9 kb length and encompassed exon 1 to 18 of the human LDLR gene (Figure 1).

At hybridization, all probes were labelled with 32P-dCTP at 3000 Ci/mmol (Amersham International) by nick translation or the multiprime method, and 2.0 x 10^8 to 1.0 x 10^9 cpm/μg was measured as the specific activity of probes. Serum lipid levels were measured by the enzymatic method with overnight fasting blood samples. Lipoproteins were separated by sequential ultracentrifugation. Cord blood samples were taken carefully by an obstetrician to avoid contamination of the mothers' blood. Each patient was studied after informed consent and approval by the Committee on New Drugs and Equipment at Kanazawa University Hospital.

Results

The LDLR genes of 35 unrelated heterozygous FH patients were analyzed with the Southern blotting method by using a cDNA probe, pLDLR-2HHI. Only two patients with FH (KY and SO) showed an abnormal fragment pattern (Figure 2). A normal LDLR gene shows one hybridized fragment about 16.0 kb in length after BamHI digestion. Two fragments, one the same size as normal and the other approximately 6 kb smaller, were found, however, on the lanes of KY and SO. The filter shown in Figure 2 was rehybridized with a full length cDNA, pLDLR-3XSI. There were no other differences except the abnormal 10.0 kb fragment (data are not shown). With PvuII digestion added to BamHI digestion, the abnormal fragment that is 6 kb smaller than normal was detected again. We therefore concluded that these two patients had a common mutant LDLR allele with a 6 kb deletion.

To confirm this, the family members of KY and SO were studied. In KY's family, DNA samples digested with BamHI digestion and BamHI-PvuII double digestion hybridized with pLDLR-2HHI. With BamHI, KY and SO have abnormal fragments approximately 10.0 kb in length. The difference in fragment size between the abnormal fragment and the normal one, 6 kb, is identified after BamHI-PvuII double digestion in the same way as after BamHI digestion.

Figure 1. Restriction map of a full-length cDNA for the human LDLR (pLDLR-3) and cDNA probes used in this study. The black line represents the vector of this clone. The wide, open bar indicates the coding region, and the narrow portions indicate the noncoding regions. The solid boxes represent the Alu-repeat sequences. B = BamHI site, S = Smal site, X = XbaI site.

Figure 2. Detection of an abnormal fragment of LDLR gene with a 6-kilobase deletion. Autoradiograph after BamHI digestion and BamHI-PvuII double digestion hybridized with pLDLR-2HHI. With BamHI, KY and SO have abnormal fragments approximately 10.0 kb in length. The difference in fragment size between the abnormal fragment and the normal one, 6 kb, is identified after BamHI-PvuII double digestion in the same way as after BamHI digestion.
tion (Figure 4). Her husband was normocholesterolemic and showed a normal fragment pattern, PvuII genotype AA30 (data are not shown). Thus, we believe that MT and CM had inherited from SO a mutant gene that produced FH.

To determine the localization of this 6 kb deletion, the genomic DNA of SO was digested by several restriction enzymes (Figure 5A). Additional KpnI digestion to BamHI digestion caused the disappearance of the abnormal fragment visualized with only BamHI. This result indicated that the KpnI site in intron 14 was preserved and almost all parts of exon 15 had been deleted in this mutant gene. This consideration was supported by the analysis with BglII or EcoRV (data are not shown). There was no difference in the abnormal fragment size between BamHI digestion and BamHI-HindIII double digestion. Thus, the HindIII site in intron 15 is thought to be missing in this mutant gene. XbaI-BamHI double digestion also produced an abnormal fragment that was 6 kb smaller than the normal one, but showed no other differences when compared to the normal control. This indicated that the XbaI site at the 3' end of intron 15 had also been preserved. All these data and other results with PvuII suggested that this 6 kb deletion was located between the KpnI site in intron 14 and the XbaI site in intron 15. In other words, it encompassed an area near the 5' end of exon 15 up to the HindIII site in intron 15 (Figure 5B).

The two daughters of SO were pregnant when this abnormal LDLR gene was identified and thus the lipid levels and the LDLR genes of cord blood samples from both babies were examined at birth. The serum and LDL cholesterol levels of MT's baby were 78 mg/dl and 31 mg/dl, respectively (Table 1). On Southern blotting analysis, she revealed an abnormal 10.5 kb fragment (Figure 4). Conversely, CM's baby was normocholesterolemic (serum cholesterol level was 51 mg/dl and LDL cholesterol level was 13 mg/dl) and showed a normal fragment pattern, PvuII genotype AA30 (Figure 4).

Discussion

In this study, the LDLR genes of 35 heterozygous FH patients were screened and a 6 kb deletion was found in two unrelated patients. Judging from the results of family studies of both patients, we conclude that the deletion of the LDLR gene results in FH. This deletion was located between the KpnI site in intron 14 and the XbaI site near the 3' end of intron 15 (Figure 5B). This indicates that the mutant gene eliminates only exon 15, an exon which encodes the O-linked sugar domain of LDLR.

Lehrman and colleagues have reported two cases with large deletions in the LDLR gene. In one case of internalization defective homozygous FH, Alu-Alu recombination produced the 5 kb deletion including the carboxyl terminal of the O-linked sugar domain and the membrane spanning

![Pedigree of K.Y.](https://example.com/figure3.jpg)

**Figure 3.** The analysis of LDLR gene in the family of KY. In all patients with heterozygous FH (C, O), the abnormal fragments (10.0 kb) are found after digestion by BamHI hybridized with pLDLR-2HHI. Non-FH members (O) do not show this abnormal fragment.

![Pedigree of SO.](https://example.com/figure4a.jpg)

**Figure 4.** The analysis of the LDLR gene in the family of SO. A. In the pedigree, O indicates patients with heterozygous FH, and ◯ and □ indicate non-FH family members. B. The autoradiograph after PvuII digestion hybridized with pLDLR-2HHI. All three patients (SO, MT, and CM) in this family have a common abnormal 10.5-kb fragment derived from a mutant allele. MT's baby has three fragments (14.0, 3.5, and 2.5 kb) derived from PvuII allele B, and an additional 10.5-kb abnormal fragment like MT. CM's baby shows a normal fragment pattern that suggests a PvuII genotype AA30.
domain. The other case, JD's mother, had been diagnosed for null type and exon-Alu recombination deleted 5 kb that encompassed exon 13 to 15. Upon comparison with other reported data, we concluded that the 5 kb deletion shown here was a new variant of LDLR gene.

Hobbs and colleagues reported a case with a large deletion of the LDLR gene. This deletion had removed only one exon, exon 5, and had been produced by the interstrand Alu-Alu recombination. Lehman and colleagues analyzed two deletions of LDLR gene considered as the products of the intrastrand Alu-Alu recombination and another deletion produced by the interstrand recombination. The deletion reported here might also have been produced by Alu-Alu recombination, because the 3' end of the deletion was near the Alu sequence in intron 15 (Figure 5B).

Davis and coworkers reported that the deletion of the clustered O-linked sugars produced by the transfection of artificially deleted cDNA did not affect the binding, internalization, or recycling of the LDLR. Since the splice sites for the 3' end of exon 14 and the 5' end of exon 16 interrupt the same nucleotide position within a codon, the splicing of exon 14 to exon 16 should not interrupt the reading frame. Thus, the gene mutation that selectively deletes the exon 15 should lead to the production of LDLR lacking the O-linked sugar domain. Thus, the analysis of this abnormal LDLR gene product will provide important results connected to the function of this domain.

The early diagnosis of FH and the reduction of LDL cholesterol levels are prerequisites for preventing coronary artery disease. Prenatal diagnosis of receptor-negative homozygous FH has been established by the assessment of LDLR activity with cultured amniotic fluid cells. About 50% of their LDLR activity allows us to make a prenatal diagnosis of heterozygous FH. There is, however, some risk involved in obtaining the amniotic fluid cells. Thus, a prenatal diagnosis of FH cannot be made in every pregnancy for all FH patients. Neonatal diagnosis of FH is usually carried out by measuring the total cholesterol and LDL cholesterol levels of cord blood samples. This method, however, is not so reliable for identifying FH patients in the general population, and an accurate identification of FH can only be done after 1 year of age.

In SO's family, MT's baby showed the same abnormal 10.5 kb fragment as her mother. Unfortunately, we were unable to analyze the LDLR gene of her father, but this baby had inherited from her father the normal allele (PvuII allele A) that was cleaved at a polymorphic PvuII site and the 6-kb deleted mutant gene of SO. The blood samples from adults were peripheral blood; from infants, samples were from cord blood.

Table 1. Lipoprotein Cholesterol Levels of SO and Members of Her Family

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Total</th>
<th>VLDL</th>
<th>IDL</th>
<th>LDL</th>
<th>HDL</th>
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<tbody>
<tr>
<td>SO</td>
<td>54</td>
<td>315</td>
<td>6</td>
<td>8</td>
<td>204</td>
<td>62</td>
</tr>
<tr>
<td>Husband</td>
<td>57</td>
<td>164</td>
<td>6</td>
<td>2</td>
<td>91</td>
<td>51</td>
</tr>
<tr>
<td>MT*</td>
<td>26</td>
<td>389</td>
<td>33</td>
<td>28</td>
<td>245</td>
<td>48</td>
</tr>
<tr>
<td>CM*</td>
<td>25</td>
<td>393</td>
<td>16</td>
<td>48</td>
<td>281</td>
<td>34</td>
</tr>
<tr>
<td>Control† (n = 17)</td>
<td>173 ± 12</td>
<td>10 ± 16</td>
<td>11 ± 7</td>
<td>94 ± 22</td>
<td>57 ± 11</td>
<td></td>
</tr>
<tr>
<td>MT's baby</td>
<td>0</td>
<td>78</td>
<td>4</td>
<td>31</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>CM's baby</td>
<td>0</td>
<td>51</td>
<td>7</td>
<td>19</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Control† (n = 12)</td>
<td>59 ± 14</td>
<td>2 ± 2</td>
<td>21 ± 8</td>
<td>32 ± 7</td>
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<td></td>
</tr>
</tbody>
</table>

The results were means ± standard deviations.7,30

*Blood samples were obtained during their pregnancies.
†Results were means ± standard deviations.7,30
and LDL cholesterol levels in her cord blood were lower than the criteria for neonatal diagnosis of FH (above 41 mg/dl in LDL cholesterol and 100 mg/dl in total cholesterol) this discrepancy could be explained as follows. Non-hepatic cells, which seem likely to play a dominant role in removing LDL from the circulation in the neonatal period, might be able to function properly in spite of this gene mutation which deletes exon 15. This consideration would be consistent with previous data in fibroblasts, if the mutant genes reported here have produced the LDLR lacking O-linked sugars. On the other hand, hepatic LDLRs, which are major factors in determining serum cholesterol level in children and adults, might be impaired by the deletion of exon 15. In other words, O-linked sugars might be crucial not for nonhepatic LDLRs, but for the LDLRs in the liver.

The other case, CM’s baby, was normocholesterolemic and had a normal fragment pattern (Figure 4). Although the LDLR gene of her father was not analyzed, it is believed that this baby inherited the normal LDLR allele (PvuII allele A) from her mother, CM. We diagnosed this baby as non-FH by her normal levels of total and LDL cholesterol and the normal fragment pattern of the LDLR gene. We anticipate the diagnosis of FH with the method reported here in other members of these two families.

Both KY and SO were born in the Tonami district of Toyama Prefecture in Japan (Figure 6). We therefore refer to patients with this new mutant gene as having ‘FH-Tonami’. Although a common link between these two families could not be established, even though we traced their family histories back 100 years, results of the mutant gene analysis suggest a common ancestor with FH.

Detection of most mutant alleles of the LDLR gene is impossible by genomic Southern blotting analysis since gross structural alteration of the LDLR gene is presumably rare. In this study, only two of the 35 mutant alleles of the LDLR gene indicated large deletions detected by this method. When new molecular biological techniques that can easily detect point mutations or several nucleotides alterations become available, all mutations of the LDLR gene in FH patients can be correctly diagnosed.

Acknowledgments

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References

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